Protein phosphatase 1β is required for the maintenance of muscle attachments

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Type 1 serine/threonine protein phosphatases (PP1) are important regulators of many cellular and developmental processes, including glycogen metabolism, muscle contraction, and the cell cycle [1–5]. *Drosophila* and humans both have multiple genes encoding PP1 isoforms [3,6,7]; each has one β and several α isoform genes (α_1, α_2, α_3 in flies, α and γ in humans; mammalian PP1β is also known as PP1δ). The α/β subtype differences are highly conserved between flies and mammals [6]. Though all these proteins are >85% identical to each other and have indistinguishable activities *in vitro,* we show here that the *Drosophila* β isoform has a distinct biological role. We show that *PP1β9C* corresponds to *flapwing* (*flw*), previously identified mutants of which are viable but flightless because of defects in indirect flight muscles (IFMs) [8]. We have isolated a new, semi-lethal *flw* allele that shows a range of defects, especially in muscles, which break away from their attachment sites and degenerate.

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Results and discussion

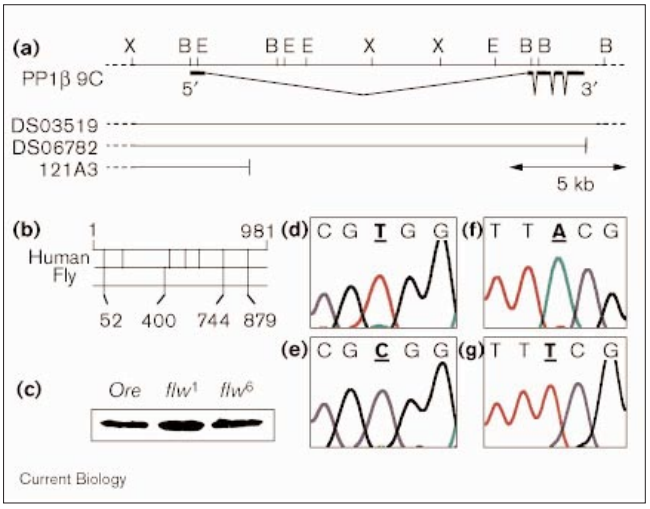
Though *Drosophila* has four genes encoding PP1 isoforms [6,7], one of these (PP1α87B) encodes 80% of the total PP1 activity [2]. Another PP1 gene, PP1β9C, is much more closely related (94% identity [6]) to mammalian PP1β than it is to other PP1 genes from flies or mammals (85% identity), suggesting that it has a conserved function distinct from that of PP1α. We have analysed the intron/exon structure of PP1β9C and find that three of the four introns in its coding region correspond exactly to three of the seven introns in the human gene, confirming the ancestral origin of this isoform (Figure 1). The α and β isoforms therefore appear to have been preserved in each lineage since their divergence >500 million years ago, despite their extreme sequence identity.

The PP1 isoforms have indistinguishable activity *in vitro*, so we attempted to identify the non-redundant role(s) of PP1β by mutational analysis. We found that *PP1β9C* corresponds to the flightless mutant *flapwing* (*flw*), which was originally isolated in a screen for viable, flightless mutants [8]. The conservation of PP1β between flies and mammals suggests an essential role. We therefore screened for lethal alleles of *flw* using the chemical mutagen ethyl methane sulphonate (EMS). We successfully isolated flw^6, a new, semi-lethal allele of *flw*. This demonstrates for the first time that the α and β isoforms are both essential, despite their 85% sequence identity. The flw^1/flw^6 flies are completely flightless and their muscle and wing phenotypes resemble combinations of flw^1 with deficiencies covering the *flw* region (flw^1/*Df(1)N110* and flw^1/*Df(1)Hk*), so flw^6 appears to act as an amorphic allele.

The IFMs comprise two sets of muscles, the dorsal longitudinal muscles (DLMs) and the dorsoventral muscles (DVMs). Alternating contraction of these two sets of muscles resonates the thorax, which in turn drives the wings. The development of the IFMs has been analysed in some detail [9]. They are constructed during pupation from myoblasts, previously sequestered in the wing imaginal disk. The DLMs are built on a template of the larval oblique muscles, which are spared from the general histolysis of larval muscles, whereas the DVMs are formed by *de novo* fusion of myoblasts. We investigated the IFM defect in *flw* mutants using polarised light microscopy (Figure 2), dissection and plastic sections (data not shown). The flw^1 flies show a variable phenotype ranging from normal, through disorganised to absent IFMs. This phenotypic range was equally broad in an isogenic derivative line. IFMs were never present in flw^1/*Df(1)N110*, flw^1/*Df(1)Hk* or flw^1/flw^6 flies, but the jump muscle (tergal depressor of trochanter muscle or TDT) appeared normal. In the few (<1%) flw^6/Y males that eclosed, the IFMs were absent and the TDT disorganised. All aspects of the flw^6/Y phenotype were fully rescued by a *PP1β9C* cDNA expression construct, demonstrating that *flapwing* corresponds to PP1β9C (Figures 2,3).

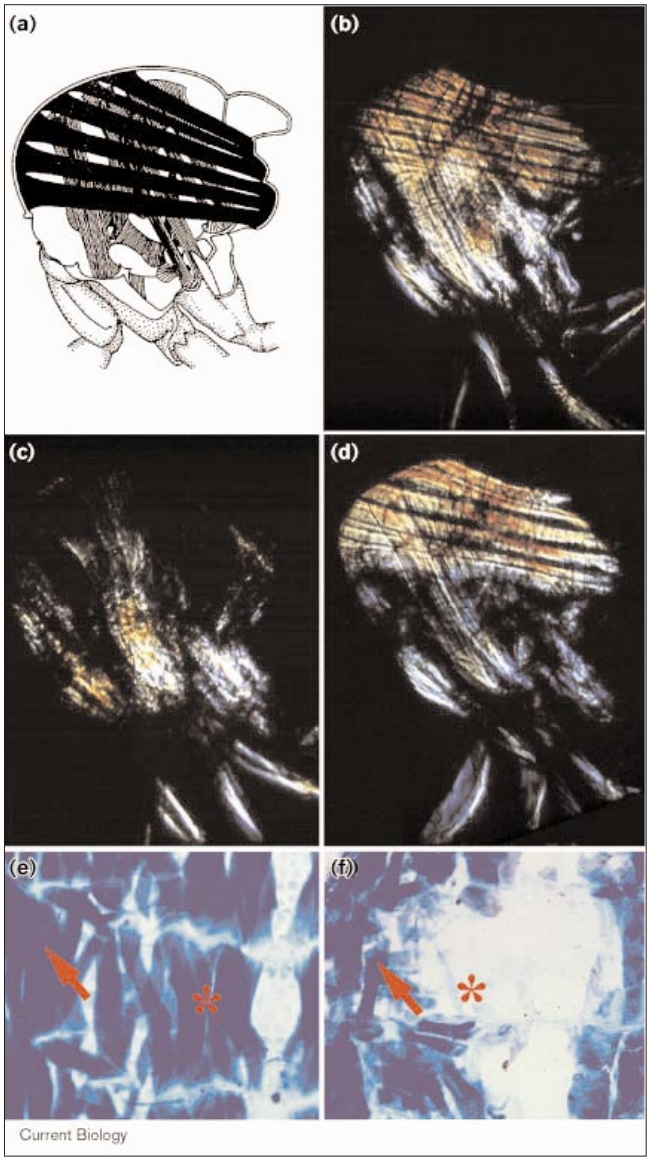
We analysed IFM development in flw^1/*Df(1)Hk* pupae. The early development of the IFMs appeared normal: the pretemplates on which the DLMs form were present, the DVMs also developed normally. Muscle defects became

Figure 1



Molecular characterisation of *PP1β9C*. **(a)** Gene structure of *PP1β9C*. Sites for the restriction enzymes *Bgl*II (B), *Eco*RI (E) and *Xho*I (X) are marked. DS03519 and DS06782 are P1 clones from the Berkeley *Drosophila* genome project, 121A3 is a cosmid from the European *Drosophila* Genome Project. **(b)** Comparison of the intron/exon structure of human and fly PP1β within the 981 nt coding region. The nucleotide positions of the four fly introns are shown; three of them correspond exactly with introns in the human homologue. **(c)** Western blot of pupal proteins probed for PP1β. Similar levels of PP1β protein are present in wild-type and mutant pupae. **(d–g)** Sequences of mutated regions in *flw*^{1} and *flw*^{6}, with traces from an automatic sequencer below. (d,e) At the codon corresponding to Val284 (GTG) in (d) the wild type, (e) *flw*^{1} has GCG, encoding Ala. (f,g) At the codon corresponding to Tyr133 (TAC) in (f) the wild type, (g) *flw*^{6} has TTC, encoding Phe.

Figure 2

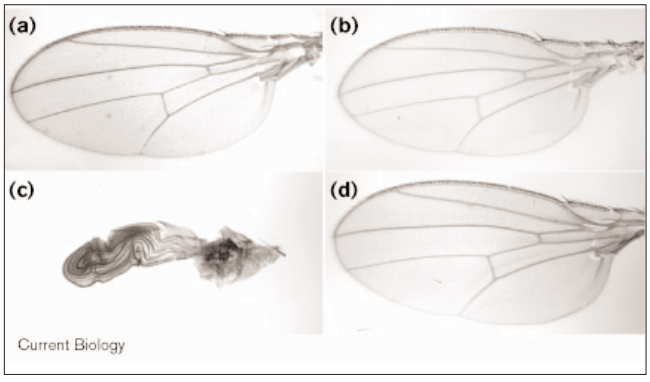


Muscle defects in *flw*^{6} males. **(a–d)** IFMs; anterior is left, dorsal is uppermost. (a) Diagram of thoracic musculature (reproduced from [21] with permission). Black, DLMs; grey, DVMs. (b–d) Polarised light micrographs showing thoracic muscles of (b) wild-type, (c) *flw*^{6}/Y, and (d) *flw*^{6}/Y; *P[hsp26-PP1β9C]*/+ males. The latter express *PP1β9C* from the *hsp26* promoter. IFMs are completely absent in (c) and as wild type in (d). **(e,f)** Larval body wall muscles (stained blue in a histochemical reaction for β-galactosidase expressed by a myosin heavy chain–LacZ reporter gene) of (e) wild-type and (f) *flw*^{6}/Y males. Arrows point to poorly attached, remnant muscles in the mutant (f) and to the corresponding region in (e). Asterisks mark a region devoid of muscles in the mutant (f) and the corresponding region in (e). Anterior is to the top and the dorsal midline is right of centre.

apparent at about 28 h after puparium formation. In the wild type, the muscles shorten and then elongate to form the final structure as they send out processes to their attachment sites. In the mutant, most of the muscles broke away from their posterior attachment sites and were found as ball-like structures at the anterior segment boundaries. These clumps were dissected and examined by electron microscopy. The sarcomere organisation appeared normal (data not shown).

Less than 1% of *flw*^{6}/Y animals survive to adult at 25°C. The period of lethality extends from the second larval instar onwards. Dying larvae are very sluggish, suggesting a possible defect in the larval body wall musculature. In the abdominal segments of the larva (A1–A7), each hemisegment contains a stereotyped set of 30 muscles [10]. We examined the musculature of *flw*^{6}/Y under polarised light and by using a muscle-specific LacZ marker. The muscle pattern in these larvae was clearly disrupted (Figure 2e,f). Many muscles are missing, and others floated away during dissection, indicating very weak attachment. We suggest that this muscle attachment defect leads directly to the semi-paralysed phenotype of the dying larvae and causes death by an inability to feed. Adult escapers also have very poor ambulatory ability, but we have not studied the muscle attachments at this stage. Since the muscles appear to develop normally, but then detach, we suggest that PP1β9C is required for the maintenance, rather than the formation of muscle attachments.

Figure 3



Wing defects in *flw* mutants. **(a)** Wild type; **(b)** *flw*^{1}/Y; **(c)** *flw*^{6}/Y; **(d)** *flw*^{6}/Y ; *P[hsp26-PP1β9C]*/+. Wings of *flw*^{6}/Y flies are severely crumpled and/or blistered; this defect is completely rescued by the PP1β9C expression construct.

PP1β is the major isoform in rabbit skeletal muscle and so is the main isoform complexed to the myosin-targeting or M subunit of PP1 [11]. This complex is thought to dephosphorylate myosin regulatory light chain (MLC), antagonising myosin regulatory light chain kinase (MLCK). We considered whether the phenotype of *flw* mutants might be due to a failure to dephosphorylate Mlc-2, but *Mlc-2* mutants do not show defects in muscle attachment [12,13]. A S66A/S67A non-phosphorylatable mutant of Mlc-2 [13] does not suppress any *flw* mutant phenotypes (data not shown).

Though PP1α87B is the major isoform overall [2], it might be that PP1β9C is the only or major isoform present in muscles. The *flw* phenotype might then be due to a reduction in the overall PP1 activity in the muscle, rather than any isoform-specific role of PP1β9C. We therefore measured the PP1 activity of muscle extracts from various PP1 mutants. The total PP1 activity of larvae homozygous for *Pp1-87B*^{1} (also known as *Su-var(3)6*^{01} [1,2]) is correspondingly reduced to about 20% of that of wild type and that of heterozygotes to about 50% of wild type. We found that the PP1 activity from extracts of larval body wall muscles of these genotypes was reduced by a similar proportion (Table 1), showing that PP1α87B is the major PP1 gene in these muscles. In contrast, the PP1 activity of equivalent extracts from *flw*^{1}/Y or *flw*^{6}/Y larval body walls were only slightly lower than wild-type controls (92% and 84% of wild type). Similar results were obtained from IFM extracts from these genotypes (Table 1). *Pp1-87B*^{1} homozygous and heterozygous larvae do not show the muscle detachment phenotype of *flw*^{6}/Y larvae, nor do the adults show the IFM loss of *flw*^{1} and *flw*^{6} (data not shown). These phenotypes are therefore due to a specific requirement for PP1β9C and not to loss of overall PP1 activity in the affected muscles.

PP1β9C is required for the maintenance of muscle attachments. We investigated whether PP1β9C is required in the muscle, the epidermis or elsewhere. Using the GAL4-UAS expression system, we placed a PP1β9C cDNA under the control of a muscle-specific or an epidermis-specific promoter (Gal4-24B and Gal4-69B respectively [14]). Neither of these rescues *flw*^{6}, but a combination of the two does. This suggests that PP1β9C is required on both sides of the muscle attachment site.

In addition to the muscle attachment defects, strong *flw* mutants show extensive blistering and/or crumpling of the wing (Figure 3). The wing forms from two sheets of cells; blistering is due to a failure of these two sheets to adhere to each other. PP1β is therefore required for cell adhesion in non-muscle tissues as well as in the maintenance of muscle attachments.

Table 1

PP1 activities of muscle extracts from wild-type and mutant larvae and adults.

	Larval body wall muscles specific activity (mU/mg ± SD)		Adult IFM specific activity (mU/mg ± SD)	
	Total	PP1	Total	PP1
Oregon R	29.51 ± 1.15 (6)	24.86 ± 1.17 (4)	23.68 ± 2.52 (6)	21.02 ± 0.77 (4)
flw^{1}	29.36 ± 1.92 (6)	22.84 ± 1.05 (4)	22.68 ± 0.97 (6)	19.42 ± 0.62 (4)
FM7c/Y	30.79 ± 1.85 (6)	24.40 ± 1.65 (4)	N.D.	N.D.
flw^{6}/Y	26.85 ± 3.08 (6)	20.56 ± 2.27 (4)	N.D.	N.D.
Pp1-87B^{1}/*Pp1-87B*^{1}	9.51 ± 0.45 (6)	7.38 ± 0.18 (4)	8.85 ± 0.29 (6)	6.89 ± 0.39 (4)
Pp1-87B^{1}/+	13.86 ± 0.68 (6)	10.97 ± 0.85 (4)	12.37 ± 0.80 (6)	10.50 ± 0.77 (4)

Mean PP1 activity and total phosphatase activity is shown with standard deviation and (in parentheses) number of measurements. N.D., not done because *flw*^{6}/Y males have few or no IFMs. PP1 activities of both sets of muscle extracts are clearly lower in *Pp1-87B*^{1} homozygotes or heterozygotes than in either *flw* mutant, yet neither show the muscle detachment phenotypes of *flw* mutants. PP1 activity of *flw*^{6}/Y body wall muscle extract is significantly lower than that of extract from FM7c siblings (one-tailed two-sample *t* test, $0.05 > p > 0.01$).

Normal levels of PP1β9C protein are produced in *flw*^{1} and *flw*^{6} (Figure 1c). We therefore analysed the organisation of the PP1β9C gene and sequenced the coding region and intron/exon boundaries in each mutant. Each has a single amino acid change (Figure 1d–g). Both mutations affect amino acids that are completely conserved across all four fly PP1 genes, and indeed through to mammals. Based on the published X-ray structure of PP1 [15,16], Y133 forms a hydrogen bond to the peptide backbone of the substrate. Flw^6 protein is therefore predicted to bind all substrates with reduced affinity. V284 is in a hydrophobic pocket adjacent to the binding site for a set of regulatory proteins [17,18]. Flw^1 protein is therefore predicted to have altered affinity for these regulatory proteins. We have conducted a two-hybrid screen for PP1β9C binding proteins (D. Bennett and L.A., unpublished observations). Of 10 such proteins tested, 5 failed to bind to a V284A mutant derivative of PP1β9C, which mimics the *flw*^{1} mutation.

The PP1 gene family encodes a set of closely related proteins. Despite their similarity in sequence and *in vitro* activity, subtle sequence differences between different isoforms are conserved between flies and humans [6]. Of the mammalian genes, functional analysis by gene knockout has so far only been performed for one PP1 isoform, PP1γ [19]. This knockout eliminated both the widely expressed PP1γ1 and the testis-specific PP1γ2. Homozygous mutant female mice were viable and fertile; homozygous mutant males were viable but sterile, with a range of defects in spermatogenesis. Presumably the somatic functions of PP1γ are redundant with PP1α and/or the less closely related PP1β. We show here for the first time an essential, *in vivo* role for a minor PP1 isoform. The recent demonstration of differential subcellular distribution of the mammalian isoforms [20] suggests that this may be mediated by isoform-specific targeting and regulatory subunits, localising the isoforms to different subcellular compartments and substrates.

Supplementary material

Supplementary material including additional methodological details is available at http://current-biology.com/supmat/supmatin.htm.

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